Bacterial copper storage proteins

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Copper is essential for most organisms as a cofactor for key enzymes involved in fundamental processes such as respiration and photosynthesis. However, copper also has toxic effects in cells, which is why eukaryotes and prokaryotes have evolved mechanisms for safe copper handling. A new family of bacterial proteins uses a Cys-rich four-helix bundle to safely store large quantities of Cu(I). The work leading to the discovery of these proteins, their properties and physiological functions, and how their presence potentially impacts the current views of bacterial copper handling and use are discussed in this review.

Copper in biology

The utilization of metals by biological systems is highly paradoxical. On the one hand, metal ions provide proteins access to chemistry that would otherwise be impossible using the organic reactions that can be catalyzed by amino acid side chains. On the other hand, many of these metal ions can be toxic to cells. Copper is essential for most organisms as the cofactor for key enzymes involved in important processes such as respiration and photosynthesis (1–7). Ideas about the cellular toxicity of copper have developed in recent years, from solely being attributed to the generation of reactive oxygen species (ROS)[2] (8–11). An emerging mechanism appears to be driven by the ability of copper to bind tightly at the active sites of metalloenzymes, particularly those containing iron-sulfur clusters. This not only destroys the reactivity of the mis-metallated protein but releases iron that can produce ROS (9). This toxicity is the reason why aquated ("free") copper ions should not exist in cells and that copper is predicted to be highly restricted in eukaryotes (12) and prokaryotes (13). Copper availability appears to be largely constrained by the use of high-affinity sites in proteins (12–14), although "pools" of copper bound by other molecules are important (4, 5, 11, 15–18).

Approaches used by cells to enable safe copper handling, referred to as copper homeostasis, include sensors, transporters, chaperones, and insertion proteins with high affinity and specificity for copper (3–7, 12–14, 19–22). A well-characterized family of copper-homeostasis proteins are the copper-transporting P-type ATPases, which can remove this metal ion from the cytosol (4–7, 20–24). In eukaryotes, these copper-efflux pumps work with a cytosolic copper metallochaperone (ATOX1 in humans and Atx1 in yeast) to facilitate import into the trans-Golgi network for secreted copper enzymes (4, 5, 19, 24, 25). The two Cu-ATPases in humans (ATP7A and ATP7B) can relocate to the plasma membrane to remove excess intracellular copper when necessary (4, 24). In bacteria, the production of the copper-efflux pump CopA (23) is controlled by transcriptional regulators (sensors) such as CueR (13) and CsoR (26). CopA can work either alone or in concert with the ATOX1/Atx1 homologue CopZ to remove cytosolic copper (5–7, 20–23, 27, 28). It has recently been found that in bacteria not previously thought to possess this copper metallochaperone, such as *Escherichia coli*, CopZ can be made from the CopA gene by "programmed ribosomal frameshifting" (29).

It is emerging that the human immune system uses the toxicity of copper to attack invading pathogens. Previous minireviews in the "Thematic Series on Metals in Biology" have discussed copper biochemistry (3, 22), emphasizing its role in pathogenicity (30–34). We will therefore only touch on this issue briefly toward the end of our minireview. The main topic here is the recently discovered ability of bacteria to safely store copper using a highly novel approach (35). The more widespread and abundant class of the new family of bacterial proteins that can perform this function is cytosolic (36). This is somewhat controversial, as a widely accepted view is that bacteria have evolved not to use cytosolic copper enzymes as a way to help avoid the potential toxicity associated with their metalation (6, 13, 37).

Discovery of a new bacterial copper storage protein and its characterization

Eukaryotes are able to store cytosolic copper using metallothioneins (MTs) (38–41). Related proteins have been characterized in pathogenic mycobacteria (42), but the idea that bacterial copper storage systems could be more common was unknown. This changed with the discovery of a new family of copper storage proteins, the Csps, in the methane-oxidizing bacterium (methanotroph) *Methylosinus trichosporium* OB3b (35). It is not surprising that such a finding about copper biochemistry was made in methanotrophs as these Gram-negative

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This article contains Figs. S1 and S2 and supporting Refs. 1–3.

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[2] The abbreviations used are: ROS, reactive oxygen species; MT, metallothionein; Csp, copper storage protein; pMMO, particulate methane monooxygenase; sMMO, soluble methane monooxygenase; Mbn, methanobactin; Tat, twin-arginine translocase; BCS, bathocuproine disulfonate; PDB, Protein Data Bank.

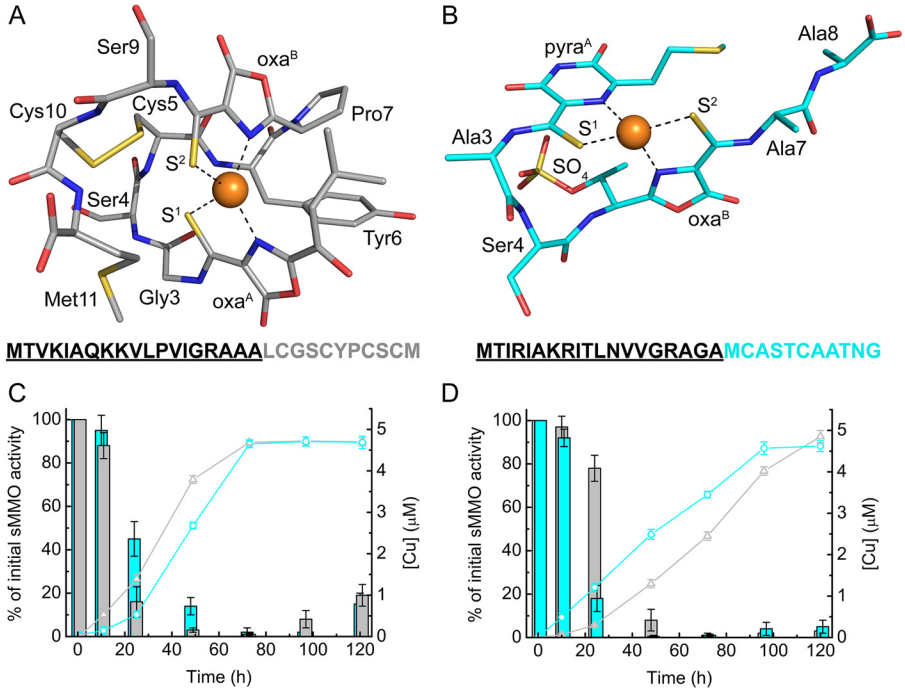

Figure 1. Structures of Cu(I)–Mbns and Mbn-mediated copper uptake. *A* and *B*, the crystal structures of the Cu(I)–Mbns from *M. trichosporium* OB3b (*A*, PDB file 2XJH) (54) and *Methylocystis hirsuta* CSC1 (*B*, PDB file 2YGI) (52). Below the structures are the sequences of the leader (*black* and *underlined*) and core peptides that make up MbnA. Core peptides are modified to give the Mbn, and the *M. hirsuta* CSC1 Cu(I)–Mbn structure is of a form with the three C-terminal residues cleaved (amino acids are numbered according to the sequence of the core peptides). The Cu(I) ions are shown as *orange spheres* ligated by the sulfur atoms (S^1 and S^2) from thioamide/enethiol groups, and two oxazolone (*oxa*) ring nitrogens in *M. trichosporium* OB3b Cu(I)–Mbn, with the N-terminal coordinating heterocycle being a pyrazinediol ($pyra^A$) in *M. hirsuta* CSC1 Cu(I)–Mbn. Other differences include a sulfate-modified Thr side chain in *M. hirsuta* CSC1 Cu(I)–Mbn and the overall hairpin-like structure of this Cu(I)–Mbn compared with the more compact *M. trichosporium* OB3b Cu(I)–Mbn. Also shown are copper uptake by (*lines*) and relative sMMO activity of (*bars*) *M. trichosporium* OB3b (*C*) and *M. hirsuta* CSC1 (*D*) cells after the addition of *M. trichosporium* OB3b Cu(I)–Mbn (*open gray triangles* and *gray bars*) and *M. hirsuta* CSC1 Cu(I)–Mbn (*open cyan circles* and *cyan bars*) to sMMO-active cells. In both cases, copper uptake and switchover from sMMO to pMMO is faster with the native Cu(I)–Mbn (52).

organisms use large amounts of copper to metabolize methane via the membrane-bound (particulate) methane monooxygenase (pMMO). This enzyme catalyzes the conversion of methane to methanol in almost all methanotrophs (17). pMMO, originally thought to have a dinuclear copper-active site, but which has very recently been suggested to be mononuclear (43), is housed on specialized intracytoplasmic membranes (17, 44) and can constitute a large proportion of total cellular protein. When copper levels are low, some methanotrophs (17, 45) have the ability to use the soluble MMO (sMMO), which has a dinuclear iron-active site (46). The switchover between these MMOs is copper-regulated, and more detail about this process and methanotroph classification and metabolism can be found in Ref. 17. Understanding how methanotrophs manage and use copper has immense environmental relevance due to methane being a highly potent greenhouse gas, and it is also essential for prospective biotechnological applications of these organisms and their MMOs (47–49).

The ability to utilize large amounts of copper results in methanotrophs having highly interesting copper-handling systems. This includes methanobactin (Mbn) (17, 50–52), which has been considered comparable to certain iron-binding siderophores (53), and is thus termed a chalkophore (50). Mbn is a modified peptide (Fig. 1, *A* and *B*) that is part of a highly specialized copper-uptake system, secreted to sequester this metal ion under limiting conditions (17, 50). The *mbnA* gene, which codes for leader (cleaved) and core (modified) peptides, has been identified in an operon along with proteins either shown or suggested to be involved in modification reactions, apo-Mbn export, and re-incorporation of Cu(I)–Mbn (17, 51, 55–59). Related Mbn operons are present in some non-methanotrophic bacteria (17, 51, 59). Work in our laboratory has found that Mbns bind Cu(I) with affinities in the 10^{20} to 10^{21} M^{-1} range and have Cu(II) affinities that are ~6–10 orders of magnitude weaker (52, 54, 60). We suggested oxidation could assist removal of the metal ion in cells (52), although a conformational change at the N terminus of the peptide now appears to be the most likely mechanism to promote release (60). Having characterized a range of Mbns isolated from spent media in which methanotrophs were grown at low copper concentrations (52, 54, 60), including determination of their high-resolution crystal structures (Fig. 1, *A* and *B*) and analysis of the Cu(I)–Mbn uptake process (Fig. 1, *C* and *D*) (52), understanding the fate of internalized Cu(I)–Mbn became our next aim. To try to isolate intracellular Cu(I)–Mbn, soluble extracts from the model switchover methanotroph *M. trichosporium* OB3b were separated using anion-exchange, followed by size-exclusion, chromatography, and fractions were analyzed for metals. A number of copper-containing peaks were observed, but none contained Mbn.

Although Mbn was not found within cells in these metalloproteomic studies, the observation of soluble copper pools in *M. trichosporium* OB3b extracts, whose abundance increased at higher copper concentrations, is extremely interesting. The

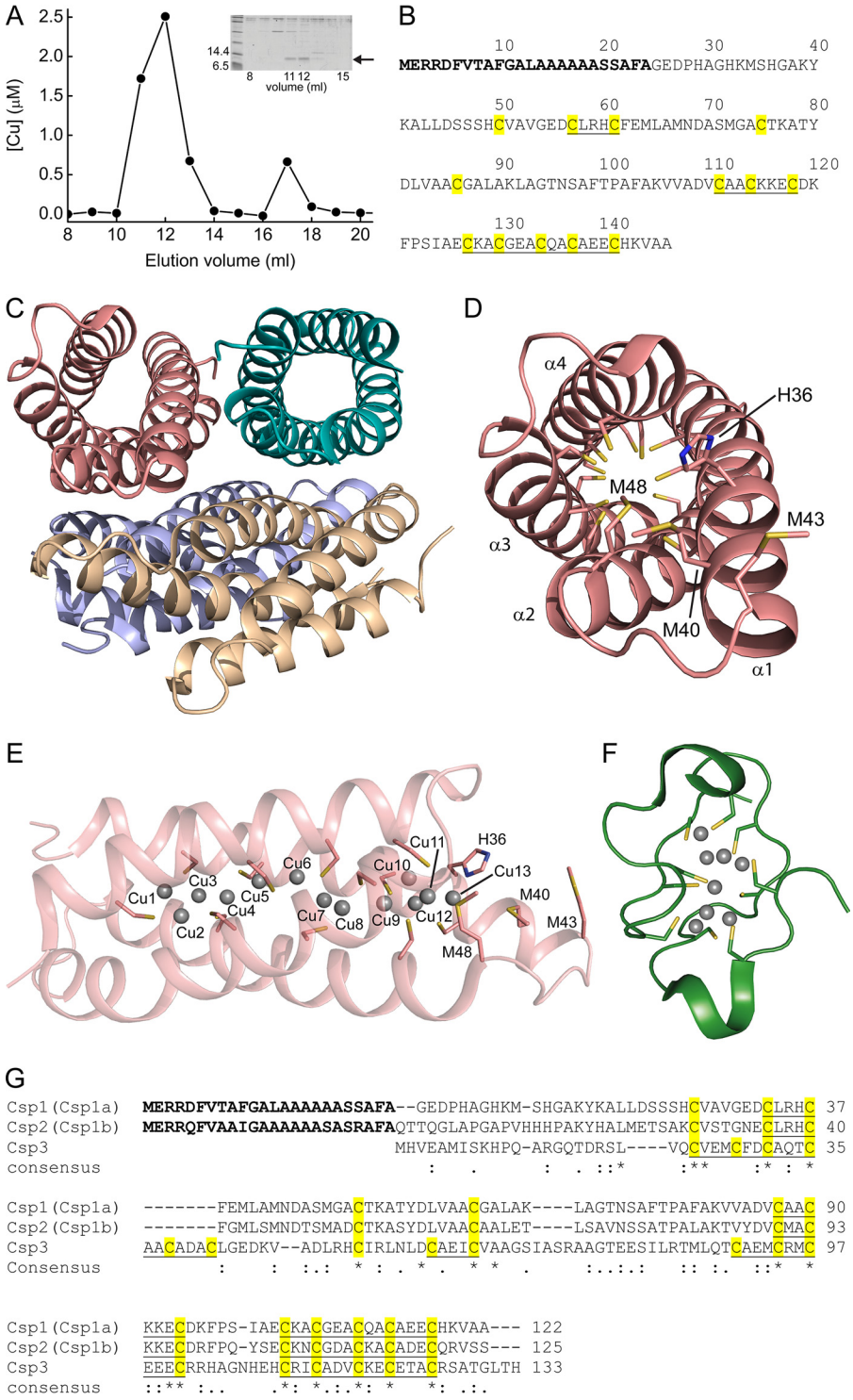

Figure 2. Discovery and characterization of Csp1 in *M. trichosporium* OB3b. *A*, copper content of anion-exchange soluble extracts of *M. trichosporium* OB3b purified on a gel-filtration column and SDS-PAGE analysis of the fractions eluted between 8 and 15 ml (35). The intensity profile of the band indicated with an *arrow* matches that of the main copper peak. *B*, sequence of this 146 amino acid residue protein (*Mt*Csp1) has a predicted Tat signal peptide (*bold*) and 13 Cys residues (*highlighted yellow*) largely present in *CXXXC* and *CXXC* motifs (*underlined*). *C*, tetrameric arrangement in the crystal structure (PDB file 5FJD) (35) of the overexpressed predicted mature form of *Mt*Csp1 (Gly-1 to Ala-122) with the *pink* (*top left*) four-helix bundle monomer (helices numbered), shown in detail (*D*), highlighting the Cys residues that all point into the core, and residues around the mouth of the bundle, in *stick* representation. *E*, structure (PDB file 5FJE) of Cu(I)-*Mt*Csp1 (35) with the metal ions represented as *gray spheres* and numbered. *F*, crystal structure (PDB file 1RJU) of a truncated form of *S. cerevisiae* MT binding eight Cu(I) ions via 10 Cys residues (39) is shown for comparison. *G*, sequence alignment of the three Csp homologues present in *M. trichosporium* OB3b created in T-coffee (65) using the predicted mature forms of *Mt*Csp1 and *Mt*Csp2.

complex mixture present in the major copper-containing fraction was further purified to identify constituent copper-binding proteins (35). Copper abundance in fractions matched the intensity of a band on an SDS polyacrylamide gel at ~12 kDa, which was purified to near homogeneity (Fig. 2*A*). The intensity profile of no other metal tested, including manganese, iron, and

zinc, corresponded with this band, identified by peptide mass fingerprinting as an uncharacterized conserved hypothetical protein possessing 13 Cys residues (Fig. 2*B*). The protein has a predicted twin-arginine translocase (Tat) signal peptide, suggesting it is folded prior to export from the cytosol (61), and cleavage is likely (62) after Ala-24 (Fig. 2*B*). Overexpressed Gly-1 to Ala-122 (no signal peptide) forms a tetramer of four-helix bundles (Fig. 2*C*), with all Cys residues pointing into the cores of the monomers (Fig. 2*D*) (35). The protein has no disulfide bonds due to the Cys residues all being found on α-helices in a fold that constrains the side chains. The protein can bind up to 12–14 Cu(I) ions per monomer *in vitro*, with an average Cu(I) affinity of $\sim 1 \times 10^{17}\ \mathrm{M}^{-1}$ (35).

In the crystal structure (35) each monomer binds 13 Cu(I) ions (Fig. 2*E*) in an unprecedented arrangement along the core of the four-helix bundle, a motif commonly found in metalloproteins (35, 63), including that binding the iron site of sMMO (46). Four of the Cu(I) ions are coordinated by two thiolates on the same α-helix in C*XXX*C motifs (Fig. 2*B*), whereas the majority of the other sites are ligated by two Cys residues on different helices. The solvent-accessible sites at the mouth of the bundle (the opposite end contains a number of hydrophobic side chains), by which Cu(I) ions are presumed to enter and leave, have different coordination environments (Fig. 2*E*) (35). This includes the binding of Cu13 by His-36 and Met-48 acting as a bridging ligand between Cu11 and Cu13. A tetramer capable of accommodating up to 52 largely solvent-protected Cu(I) ions is consistent with a role in storage, and hence the name of this novel family of copper proteins (the Csps) was devised (35). The way Csps bind Cu(I) is very different from the way a Cys-rich unstructured MT polypeptide folds around thiolate-coordinated clusters (Fig. 2*F*) (38, 39, 41). Ferritins, which store iron, are also four-helix bundles, but they use these to form a multimeric envelope that can be filled with thousands of Fe(III) ions (64). *M. trichosporium* OB3b possess three Csp homologues (Fig. 2*G*), and the first discovered, as described above, was called Csp1 (*Mt*Csp1 indicates it originates from *M. trichosporium* OB3b), and the others are named *Mt*Csp2 and *Mt*Csp3.

Comparison of Csp homologues in *M. trichosporium* OB3b

*Mt*Csp1 and *Mt*Csp2 have high identity (~60% sequence conservation), and both possess predicted (61) Tat signal peptides. The presence of two exported Csps within the same methanotroph is not uncommon (see under "Csp homologues in other methanotrophs"), where they could perform different roles (see under "Functions of Csps"). *Mt*Csp3 has no signal peptide (Fig. 2*G*), is therefore cytosolic, and has lower sequence identity to *Mt*Csp1 and *Mt*Csp2 (~20% conserved residues). Neither *Mt*Csp2 nor *Mt*Csp3 has been identified by metalloproteomics, although many of the other soluble copper-containing fractions obtained from *M. trichosporium* OB3b have yet to be thoroughly investigated. *Mt*Csp2 has not been studied due to its high sequence similarity to *Mt*Csp1 (Fig. 2*G*). *In vitro* studies of *Mt*Csp3 show it is also a tetramer of four-helix bundles having 18 Cys residues pointing into the core of each monomer (Fig. 3*A*) (36). The additional Cys residues, compared with *Mt*Csp1, are found in C*XXX*C motifs, and the protein also has no disulfide bonds. Each monomer is able to bind more metal ions within its core and has 19 Cu(I) sites in the crystal structure (Fig. 3*B*). Most of these are coordinated by two thiolates, largely alternating between Cu(I) ions bound by Cys residues from the same α-helix (in C*XXX*C motifs) and inter-helical sites. Atypical coordination is again found at the mouth of the bundle where His-110 binds Cu18 along with Cys-111, and His-104 ligates Cu19 in addition to two thiolates (Fig. 3*B*) (36). The average Cu(I) affinity of *Mt*Csp3 ($\sim 2 \times 10^{17}\ \mathrm{M}^{-1}$) is similar to that of *Mt*Csp1 (35, 36).

Important differences are found in how *Mt*Csp3 and *Mt*Csp1 bind Cu(I). In the case of *Mt*Csp3, Cu(I) binding gives rise to relatively intense fluorescence at ~600 nm upon excitation within the S(Cys) → Cu(I) ligand-to-metal charge transfer bands below 400 nm (35, 36). Such emission has been associated with Cu(I)–Cu(I) interactions in proteins binding solvent-protected Cys-coordinated Cu(I) clusters, such as the MTs (38, 42, 66). The fluorescence from *Mt*Csp3, which reaches a maximum value when it is approximately half-loaded, may be related to the formation of solvent-protected tetranuclear Cu(I) clusters within its central core (67). Similar structures do not occur in *Mt*Csp1 due to it having fewer Cys residues. Furthermore, and functionally more important, Cu(I) binding is cooperative in *Mt*Csp1 (35), but not in *Mt*Csp3 (36). This could also be related to discrete cluster formation in *Mt*Csp3 (67), but the exact cause of both of these aspects of Cu(I) binding in the Csps requires further investigation.

The most striking difference between *Mt*Csp3 and *Mt*Csp1 is the time scale of Cu(I) removal from their cores. Both have average Cu(I) affinities in the low $10^{17}\ \mathrm{M}^{-1}$ range, and assuming diffusion-controlled on-rates of $\sim 10^{8}\ \mathrm{M}^{-1}\mathrm{s}^{-1}$, unassisted Cu(I) off-rates would be extremely slow ($\sim 10^{-9}\ \mathrm{s}^{-1}$). The physiological Cu(I) acceptor for any Csp is currently unknown. As *M. trichosporium* OB3b produces an Mbn, removal by this high Cu(I)-affinity molecule (54, and *vide supra*) has been investigated (see under "Functions of Csps"). Stoichiometric concentrations of apo-Mbn removes all Cu(I) from *Mt*Csp1 in ~1 h (35), while this process takes weeks to complete for *Mt*Csp3 (36). Comparative Cu(I) removal studies have also been carried out with well-characterized chromophoric Cu(I)-chelating molecules such as bicinchoninic acid and particularly bathocuproine disulfonate (BCS). These ligands have routinely been used to measure how tightly Cu(I) binds to a range of proteins (26, 68–71), including determination of the Cu(I) affinities of Mbns (52, 54) and the average values for the Csps (35, 36). They have also been implemented as model acceptors for investigating Cu(I) removal from homeostasis proteins (68, 72). Using a large excess of the higher-affinity ligand BCS results in complete removal of Cu(I) from *Mt*Csp1 in ~1 h (35), but only ~20% from *Mt*Csp3 in 85 h (36). There is a kinetic barrier to Cu(I) removal in *Mt*Csp3, not present in *Mt*Csp1, most likely related to structural alterations at, and particularly the amino acid residues around, the mouths of their four-helix bundles, a number of which coordinate Cu(I) (Figs. 2*E* and 3*B*). Work is underway to determine the cause of this difference and to understand whether fast and slow Cu(I) removal is a distinguishing feature of Csp1s and Csp3s, respectively (see under "Functions of Csps").

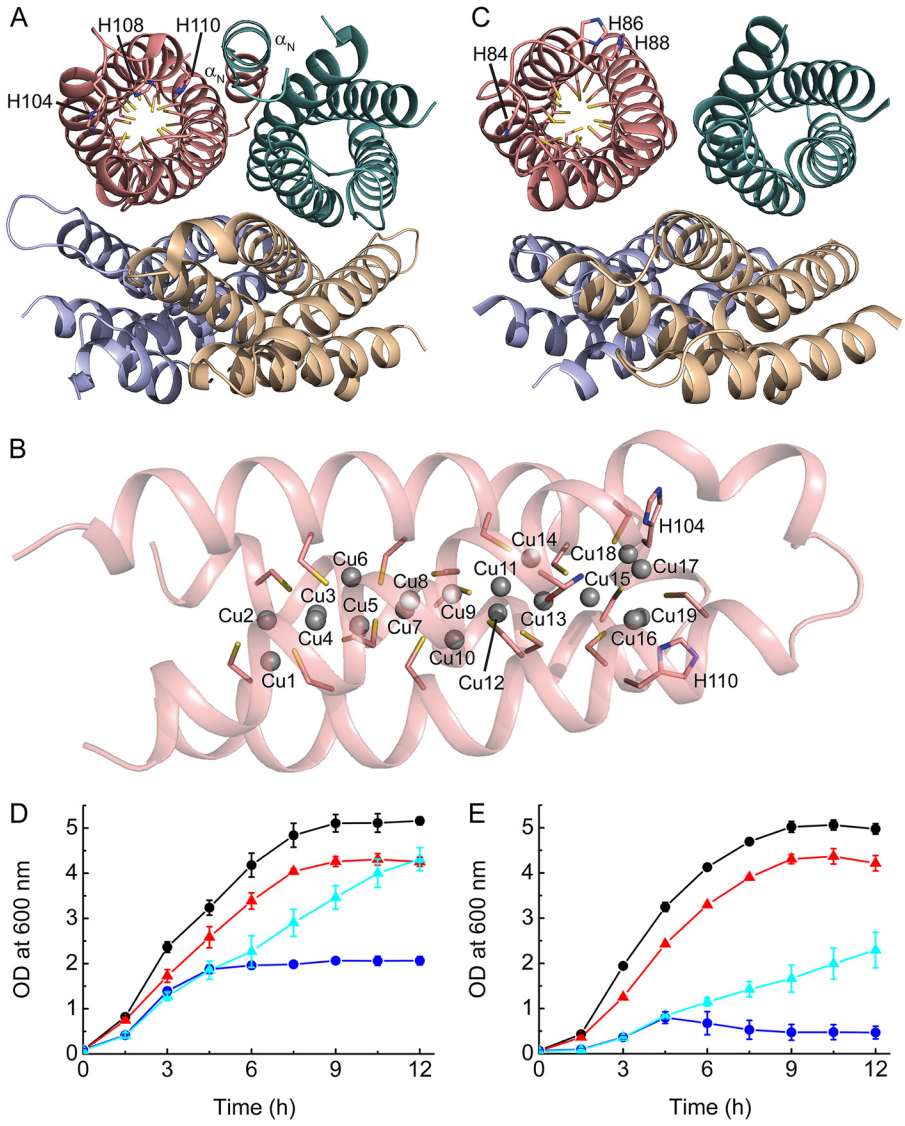

Figure 3. Structural and functional studies of Csp3s. *A*, crystal structure of the apo-*Mt*Csp3 (PDB file 5ARM) tetramer (36). The side chains of the 18 Cys residues pointing into the core and three His residues at the mouth are shown as *sticks* in the *pink* (*top left*) four-helix bundle monomer. The additional small N-terminal α-helix (α_N) is labeled in two monomers. *B*, crystal structure (PDB file 5ARN (36)) of Cu(I)-*Mt*Csp3 (α_N omitted) with the metal ions as *gray spheres* and *numbered*. *C*, crystal structure of the apo-*Bs*Csp3 (PDB file 5FIG) tetramer of four-helix bundles (36) using the same representation as in *A*, with 19 Cys residues pointing into the core of the *pink* (*top left*) four-helix bundle monomer. *D*, growth (37 °C) of Δ*copA E. coli* in the absence of (*black circles*) and plus 1.0 mM (*blue circles*) $Cu(NO_3)_2$ is compared with Δ*copA* cells overexpressing *Bs*Csp3 in the absence of (*red triangles*) and plus 1.0 mM (*cyan triangles*) $Cu(NO_3)_2$ (36). *E*, growth (37 °C) of WT *E. coli* in the absence of (*black circles*) and plus 3.4 mM (*blue circles*) $Cu(NO_3)_2$ is compared with WT cells overexpressing *Bs*Csp3 in the absence of (*red triangles*) and plus 3.4 mM (*cyan triangles*) $Cu(NO_3)_2$. Overexpressed *Bs*Csp3 can protect Δ*copA* (*D*) and WT (*E*) *E. coli* from copper toxicity.

Csp homologues in other methanotrophs

Homologues of *Mt*Csp3 are present in 34 methanotrophs whose genomes have been sequenced, and *Mt*Csp1 homologues are found in 16 (Fig. 4*A* and Figs. S1 and S2). A single *Mt*Csp3 homologue is typically found in methanotrophs having this protein (two in *Methylococcaceae bacterium* NSP1–2 and *Crenothrix polyspora*), and two *Mt*Csp1 homologues are present in over half of the sequenced methanotrophs that have this protein (*Methylocystis bryophila* appears to have three, although one of these has only seven Cys residues). A different name for a protein implies an alternative function. However, for organisms with two or more *Mt*Csp1 homologues it is not yet known whether these have distinct functions (see below). We have not established a way to differentiate between what we initially called *Mt*Csp1 and *Mt*Csp2 (35), and it may therefore be better to use Csp1a (*Mt*Csp1a) and Csp1b (*Mt*Csp1b) to signify exported Csp homologues when found within the same organism. This approach provides the clear definition that Csp1s are the exported members of this family of proteins, whereas Csp3s are cytosolic. For the purpose of this review, we will continue to use *Mt*Csp1 and *Mt*Csp2 for the exported proteins in *M. trichosporium* OB3b.

Bioinformatics also highlight residues and regions of Csp1s and Csp3s that are conserved in methanotrophs (Fig. 4, *B* and *C*, and Figs. S1 and S2). This includes the Cu(I)-coordinating Cys residues; all 13 are highly conserved in the Csp1s (Fig. 4*B*), with 15 highly conserved in Csp3s (Fig. 4*C*). The sequence of the Tat signal peptide is highly similar in the Csp1s (Fig. 4*B*). Conserved regions are found at the open end of both four-helix bundles, thought to be important for Cu(I) uptake and removal. The

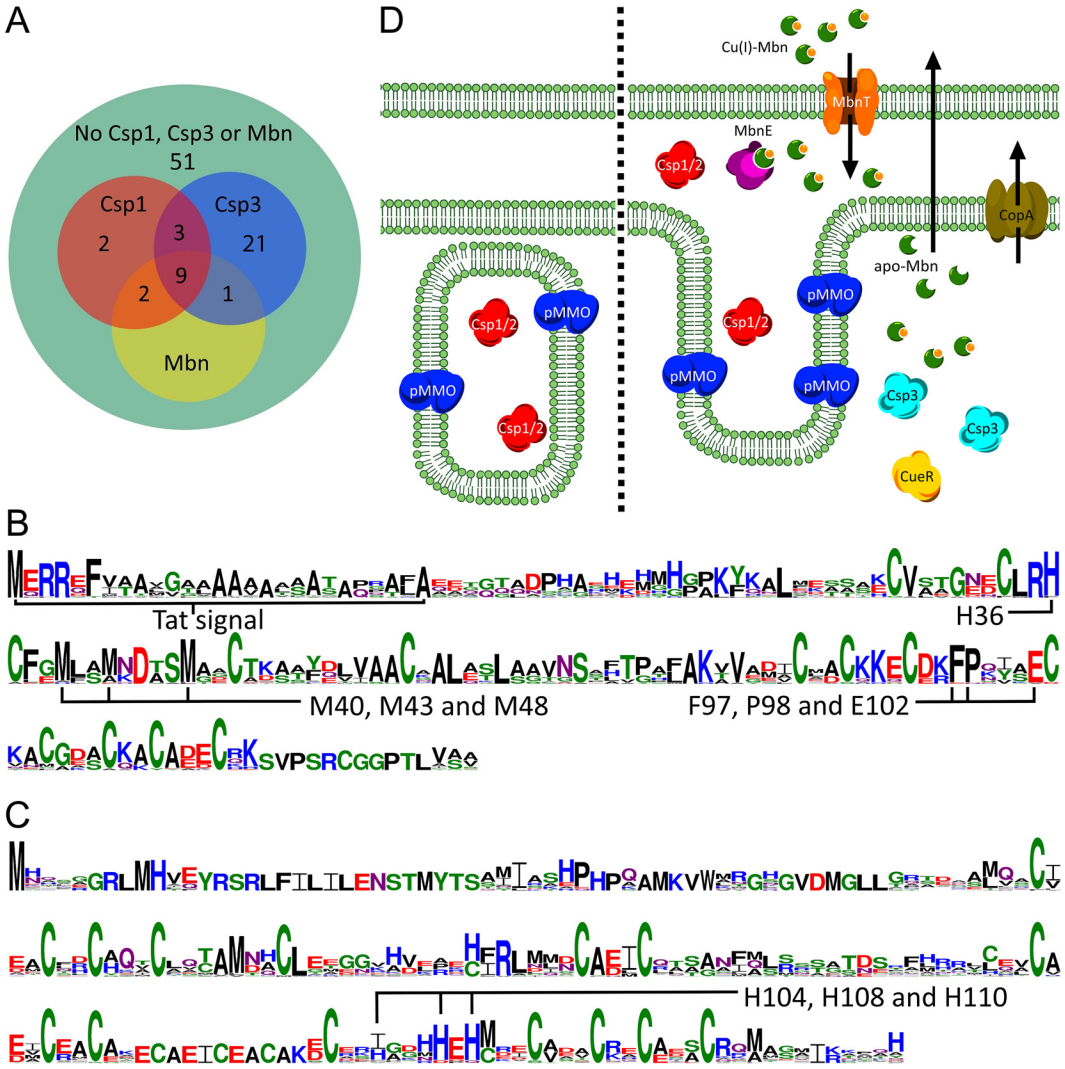

Figure 4. Bioinformatics of Csp1s, Csp3s, and the Mbn operon in methanotrophs. The 89 methanotroph genomes currently available in the NCBI database were interrogated with pBLASTp using *Mt*Csp1 and *Mt*Csp3 as search queries. *A*, Venn diagram of the distribution of Csp1, Csp3, and the Mbn operon (identified by the presence of homologues of *M. trichosporium* OB3b MbnA, MbnB, and MbnC) in methanotroph genomes. Alignments of 26 and 36 sequences (see Figs. S1 and S2) were used to produce WebLogos (73) for Csp1s (*B*) and Csp3s (*C*), respectively. In these, the overall height of the stack at a particular position represents the degree of conservation, whereas the height of the symbol for an amino acid residue (*green* for polar, *purple* for neutral, *blue* for basic, *red* for acidic, and *black* for hydrophobic) within the stack signifies relative frequency. Widths are unscaled so less frequently occupied positions in the alignment (see Figs. S1 and S2) are not represented by narrower stacks (composition adjustment was left to the default value for typical amino acid usage). Signal peptides were identified using SignalP (74) and TatP (62), and this region is labeled in *B*. *D*, schematic of the model methanotroph *M. trichosporium* OB3b showing two possible arrangements of the intracytoplasmic membranes that house pMMO and the location and potential roles of *Mt*Csp1, *Mt*Csp2, and *Mt*Csp3. The established cytosolic copper-sensing (CueR) and copper-efflux (CopA) systems, and the known locations and interactions of Mbn (MbnT is a Ton-B-dependent transporter (56) and MbnE is suggested to bind Mbn in the periplasm (58)) are also included. A much more detailed model of copper homeostasis and copper-regulated switchover in a methanotroph can be found in Ref. 59. This includes the Csps, but it does not discuss their importance.

His-36 and Met-48 ligands in *Mt*Csp1 are present in almost all homologues, along with a number of the intervening residues in the α1(His-36)–loop–α2(Met-48) region. In addition, two residues (Phe-97 and Pro-98 in *Mt*Csp1) on the loop linking α3 and α4 are highly conserved in Csp1s, as is the Glu residue (Glu-102 in *Mt*Csp1) at the start of α4 (Fig. 4*B*). Certain residues in the α3–loop–α4 region are conserved in Csp3s. This includes the His-110 ligand of *Mt*Csp3 (Fig. 3*B*) and the non-ligating His-108, but these are predominantly present only in Methylocystaceae family (*Methylosinus*, *Methylocystis*, and *Pleomorphomonas* genera) strains. His-104, which coordinates Cu18 in *Mt*Csp3, is also conserved in these strains, but is replaced by an Ile residue in most other methanotrophs that have a *Mt*Csp3 homologue (Fig. 4*C* and Fig. S2). Overall, the Cys ligands (Csp1s and Csp3s), the Tat signal peptide (Csp1s), the α1–loop–α2 (Csp1s) and α3–loop–α4 (Csp1s and certain Csp3s) regions are conserved features of these proteins in methanotrophs.

The Mbn operon is present in 12 methanotroph genomes, and due to overlap, less than half of the sequenced methanotrophs (38 of 89) possess either the Mbn operon, Csp1, or Csp3 (Fig. 4*A*). Nine methanotrophs possess all three, with none having the Mbn operon without either a Csp1 or Csp3. Overlap mostly occurs in the *Methylosinus* and *Methylocystis* genera, but there is not sufficient evidence to suggest the functions of Csp1, Csp3, and Mbn are directly related. However, the observation that Csp3 is present alone in 22 methanotrophs (Csp1 is rarely present on its own) could indicate that the func-

tion of this protein is independent of Csp1 and Mbn (see under "Functions of Csps"). Much attention has been paid to the role of Mbn in copper acquisition and utilization by methanotrophs (17, 50–52, 54–60). However, the currently identified Mbn operon is only found in just over 10% of sequenced methanotroph genomes, suggesting other systems are important for these processes. Csps are present in over 40% of these methanotrophs.

Csp homologues in non-methanotrophs

Csp3s are found in many more bacteria than Csp1s (~4000 *versus* 200, respectively, are identified using the *M. trichosporium* OB3b proteins for searches), with Csp3s present in no less than 10 different bacterial phyla and Csp1s in at least five. Approximately 140 *Mt*Csp3 homologues are found in Archaea. It has recently been claimed (75) that Csps are present in eukaryotes. However, the three proposed eukaryotic Csps (two in plants and another in a soil-dwelling fungus according to Fig. S1 in Ref. 75) are identical to bacterial Csp sequences and are therefore most likely not from the organism indicated but are due to contamination with bacterial DNA. This is not surprising given that the bacteria in question are either soil dwelling or widely distributed in the environment. The Csp1s identified in non-methanotrophs are found in Gram-negative bacteria. A notable example is found in *Neisseria gonorhoeae* (see under "Functions of Csps"), and initial studies indicate its predicted mature Csp1 has similar Cu(I) binding and removal characteristics to *Mt*Csp1.

The more prevalent and widespread nature of Csp3s raises questions about copper handling in bacteria that have this protein. Copper homeostasis has been extensively investigated in certain Csp3-possessing non-methanotrophs, with probably the best example being *Bacillus subtilis* (21, 28, 68, 76, 77). Therefore, the Csp3 from this model Gram-positive bacterium has been studied *in vitro* (36). *Bs*Csp3 is smaller than *Mt*Csp3 (108 *versus* 133 amino acids, see Fig. S2), but it has an additional Cys residue (giving a total of 19). The structure of the apoprotein, including its tetrameric arrangement, is similar to *Mt*Csp3 (Fig. 3, *A* and *C*), again with little evidence of disulfide bond formation (36). The protein binds up to ~20 eq of Cu(I) *in vitro* with an average affinity of $\sim 2 \times 10^{17}\ \text{M}^{-1}$, but removal is faster than for *Mt*Csp3 as ~85% of the Cu(I) core is acquired by BCS in 85 h (36). However, this is still very slow compared with *Mt*Csp1 (35), and the mouth of the four-helix bundle of *Bs*Csp3 shows similarities to that of *Mt*Csp3 (Fig. 3, *A* and *C*) (36). In particular, three His residues corresponding to His-104, His-108 and His-110 are present, and these are more conserved in non-methanotrophs (36). The cytosolic Cu(I) metallochaperone CopZ is present in *B. subtilis*. Removal of Cu(I) by this potential physiological partner is also slow with *Bs*CopZ acquiring ~40% of Cu(I) from *Bs*Csp3 in 64 h (36). The *in vitro* Cu(I)-binding properties and structure of *Streptomyces lividans* Csp3 are similar to those of *Mt*Csp3 and *Bs*Csp3 (36), although the average Cu(I) affinity appears to be an order of magnitude weaker (75). Crystal structures of the apo-Csp3s from *Pseudomonas aeruginosa* (3KAW) and *Nitrosospira multiformis* (3LMF) have been deposited by a structural genomics consortium. These are similar to those of other Csp3s (Fig. 3, *A* and *C*), with all Cys residues pointing into the cores of their four-helix bundle folds, and no disulfide bonds present.

Functions of Csps

The predicted Tat-exported *Mt*Csp1 acts as a copper store for methane oxidation for the following reasons. 1) copper-bound *Mt*Csp1 is isolated (35) from *M. trichosporium* OB3b grown in 5 μM copper (Fig. 2*A*) *i.e.* using pMMO to oxidize methane. None of the other metals analyzed in the metalloproteomic studies (including manganese, iron, and zinc) co-eluted with *Mt*Csp1. Furthermore, of the metal ions tested in our laboratory, only Cu(I) binds tightly *in vitro*. 2) The deletion of both genes for the exported Csps (*Mt*Csp1 and *Mt*Csp2) results in significantly faster switchover from pMMO to sMMO in *M. trichosporium* OB3b cells transferred from high to low copper (35). 3) In gene expression studies (see Fig. S5 in Ref. 58 and see also Ref. 78) *Mt*Csp1 is up-regulated in a similar manner to pMMO at a copper concentration resulting in switchover (10–12.5 μM.). 4) The copper peak and the *Mt*Csp1 SDS-PAGE band (Fig. 2*A*) are absent in sMMO-active *M. trichosporium* OB3b cells. 5) The structure of *Mt*Csp1 as a tetramer of Cys-lined four-helix bundles allows the binding of 52 Cu(I) ions. Collectively, these data provide extensive evidence that *Mt*Csp1 stores Cu(I) for pMMO allowing continued growth on methane using this enzyme when copper becomes limiting. Given the similarities of the structures and Cu(I)-binding properties of *Mt*Csp1 homologues, many having an even greater capacity for metal ions, it is almost implicit that other members of this new family of proteins are able to bind and store Cu(I).

The cellular destination of exported Csp1s depends on the cellular structure of methanotrophs (see below). The periplasmic multi-copper oxidase CueO, which is involved in copper homeostasis in *E. coli*, is also predicted to be Tat-exported (79). As this is the pathway for folded protein secretion, it had been assumed that CueO acquired the four copper ions it needs for activity in the cytosol. However, it is now thought this protein is exported in a copper-free "incomplete folding" state and acquires copper in the periplasm (79). A number of other bacterial copper proteins are predicted to be Tat-exported (7), and further work is needed to determine whether these acquire copper in the cytosol. However, it seems highly unlikely that Csp1s are exported in a partially folded state as this would potentially promote disulfide bond formation in such Cys-rich proteins, and Tat export may be required to prevent this from occurring. Csp1s therefore most likely fold completely and acquire Cu(I) in the cytosol prior to export.

Although the prevailing view is that the intracytoplasmic membranes housing pMMO are invaginations of the plasma membrane (Fig. 4*D*), most evidence is either out-dated or indirect (80–82). If these membranes are discrete from the plasma membrane (Fig. 4*D*), pMMO would be only the second example, after plastocyanin in the thylakoid compartments of cyanobacteria (83), of a bacterial cytoplasmic copper-requiring protein. If this is the case, in a methanotroph such as *M. trichosporium* OB3b having two exported Csps, *Mt*Csp1 could deliver Cu(I) to the intracytoplasmic membranes for pMMO while *Mt*Csp2 transfers Cu(I) to the periplasm for other copper-requiring enzymes (Fig. 4*D*). *Mt*Csp2 is not up-regulated by

10 μM copper (78), and this suggested function may not require copper-regulated expression (see below), or it may occur at higher copper concentrations than those causing switchover (it is also possible that *Mt*Csp2 could act as a Cu(I) store for pMMO at higher copper concentrations). In methanotrophs that have a single Csp1, we assume this will only store Cu(I) for pMMO if the enzyme is housed in cytoplasmic compartments. However, if the intracytoplasmic and plasma membranes are contiguous, a single Csp1 could store Cu(I) for pMMO and other destinations in the periplasm. As already stated, most Csp1-possessing methanotrophs also have Mbn (Fig. 4*A*), which they produce under copper-limiting conditions (17, 50), when the Cu(I) from a Csp would be required. Therefore, during switchover from pMMO to sMMO it is possible that apo-Mbn may play a role in removing Cu(I) from *Mt*Csp1, a process that readily occurs *in vitro* (35), to aid delivery to pMMO. In non-methanotrophs, Csp1 will deliver Cu(I) to the periplasm. Export of a protein that can store large amounts of Cu(I) (there is little sign of oxidation upon prolonged exposure of Cu(I)-*Mt*Csp1 in air) will provide and stabilize a source of cuprous ions outside the cytosol, which may otherwise be difficult in the more oxidizing periplasm. This could be the oxidation state of copper required for insertion into certain enzymes, as appears to be the case for pMMO.

The precise function of a cytosolic Csp3 is currently unknown, although a general role in Cu(I) storage while preventing toxicity is presumed. Bioinformatics (Fig. 4*A*), and the more widespread nature of Csp3s both in methanotrophs and other bacteria, suggest that the function of Csp3 is not directly linked to Csp1. Preliminary *in vivo* studies on the *csp3*-delete strain of *B. subtilis* show a weak, and unusual, copper-dependent phenotype (36). Growth in LB media is inhibited relative to WT *B. subtilis* in the range of ~1.5–2 mM added Cu(II), but only after cells have been grown for more than 12 h in the presence of the metal. Obtaining this phenotype reproducibly is difficult, being sensitive to growth conditions and particularly copper concentration. Transcriptional studies have shown that *Bs*Csp3 is up-regulated under spore-forming and stress-inducing conditions, including elevated NaCl concentrations (84), but the response to copper was not tested. Interestingly, both Csp1 and Csp3 are up-regulated when the methanotroph *Methylocystis* sp. SC2 is grown in 0.75% NaCl (85). The relevance of salt stress on Csp expression remains unclear. The multi-copper oxidase CotA is one of very few predicted copper enzymes in *B. subtilis* (2) and is a component of the spore coat where it is thought to be involved in pigment production (86), and *Bs*Csp3 could store Cu(I) for this enzyme. The *P. aeruginosa* Csp3 (the protein was incorrectly called a Csp1, but it does not possess a signal peptide and is cytosolic) is not induced (87) by the addition of 0.5 mM Cu(II). However, neither is CopA2, a second copper efflux pump that is not required for copper tolerance in *P. aeruginosa*, but is suggested to be involved in export coupled to copper acquisition by cytochrome *c* oxidase (88). The *S. lividans* Csp3 is up-regulated by 0.4 mM Cu(II) and in a *csoR* (copper-sensitive operon repressor) deletion mutant (89). A transcriptomic study of the Gram-negative bacterium *Sphingobium* sp. ba1 has shown up-regulation of Csp1 and Csp3 in response to 10 mM Ni(II), but under these conditions copper resistance systems, including CopA, are also up-regulated (90).

Gene expression studies show that *Mt*Csp3 is not up-regulated at the relatively low copper concentrations (10 μM) required for switchover (78). However, putative CopAs in *M. trichosporium* OB3b are also not up-regulated under these conditions. Copper detoxification is not the proposed primary function of Csp3s, but *Bs*Csp3 can provide protection against copper toxicity when overexpressed in both the *copA* delete strain (Δ*copA*) (Fig. 3*D*) (36) and also WT *E. coli* (Fig. 3*E*). In both cases, the cells overexpressing *Bs*Csp3 accumulate more copper than control cells, and Cu(I)-*Bs*Csp3 is observed. As well as being able to complement the phenotype caused by deletion of the copper-efflux pump (23), overexpressed *Bs*Csp3 provides an additional growth advantage at elevated copper to having CopA alone. Furthermore, *Bs*Csp3-bound Cu(I) can be withheld from the efflux pump. It has also been found that in *S. lividans* Csp3 enables growth at higher copper levels (75).

When considering the functional properties of Csps, it is important to keep in mind key *in vitro* results (35, 36). Csp3s can generally bind a greater number of Cu(I) ions than Csp1s, due to usually having more Cys residues (Figs. 2, *E* and *G*, 3*B*, and 4, *B* and *C*, and Figs. S1 and S2 for methanotrophs). Csp1s and Csp3s have similar average Cu(I) affinities (~10^{17} M^{-1}) yet exhibit dramatic differences in terms of Cu(I) removal rates. How Cu(I) is extracted from Csps in cells is unknown. Many copper homeostasis proteins and copper target enzymes/proteins have higher Cu(I) affinities, typically in the 10^{17} to 10^{21} M^{-1} range (13, 26, 40, 68–71), and their ability to acquire Cu(I) from Csps is thermodynamically favored. Faster Cu(I) unloading by small molecule Cu(I) ligands occurs for Csp1s (35), but a kinetic barrier to removal is present in Csp3s (36). The interplay between thermodynamics and kinetics in copper homeostasis is currently not well understood. Furthermore, how many of the proteins involved in this process acquire copper is unknown (apart from the CopZ–CopA interaction). Csp1s are expected to be exported after acquiring Cu(I), and Csp3s kinetically trap Cu(I) in the cytosol. These proteins may therefore have evolved different approaches to enable them to bind and maintain a store of Cu(I) even in the presence of proteins with higher affinities (CopZ acquires Cu(I) very slowly from *Bs*Csp3). In methanotrophs, which can have both a Csp1 and Csp3, such as *M. trichosporium* OB3b, the dramatic variation in removal rates could be more important (as may differences in Cu(I)-binding cooperativity) and suggests that the exported Csps act as a more temporary store of Cu(I) for pMMO, whereas Csp3 plays a role in longer-term storage. Whether this distinction between Cu(I) removal rates exists for all Csp1s and Csp3s has to be established. If slow Cu(I) removal is a conserved feature of Csp3s, then the requirement for a longer-term store needs to be understood, as well as how the kinetic barrier to removal is overcome when Csp3-bound Cu(I) is required.

Possible link between Csps and pathogenicity?

As mentioned in the Introduction, and covered in previous minireviews in this series, the interplay between copper homeostasis systems in a pathogen and host is beginning to be rec-

ognized as important for virulence (30–34, 91). Compared with nutritional immunity used to withhold other essential metal ions, hosts are thought to expose invading pathogens to copper (32, 33, 91). In mammalian hosts, ATP7A pumps copper into the phagolysosomal compartment, and copper homeostasis systems can protect the pathogen against this attack (30–33, 91–93). A number of possible defense approaches have been identified, such as copper efflux and sequestration, including a Cu(II)-binding siderophore (34, 94). Csps are present in pathogenic bacteria, such as *N. gonorrhoeae* (Csp1), *Streptococcus pneumoniae* (Csp3), *Salmonella enterica* sv. Typhimurium (Csp3), and the opportunistic pathogen *P. aeruginosa* (Csp3). The ability of Csps to bind large quantities of Cu(I) would make them ideal to defend pathogens against copper attack by a host. The Cu(I)-buffering ability of Csp3 that prevents toxicity in the Δ*copA* strain of *E. coli* (Fig. 3, *D* and *E*) demonstrates that these proteins, when produced at relatively high levels, can take the place of copper-efflux pumps, known virulence factors (30, 88, 92, 93), in providing protection against elevated copper levels. The only other characterized bacterial copper-storing protein is the MT-like MymT found in pathogenic mycobacteria (42), but this does not appear to be required for infection. Whether a Csp would help a pathogen fight against host-based copper attack remains to be established.

Concluding remarks

The Csps were identified in methanotrophs, bacteria with atypically high copper demands, which they use for methane oxidation, and *M. trichosporium* OB3b possesses three homologues: two closely related proteins having predicted Tat signal peptides and a cytosolic version. Exported *Mt*Csp1 stores Cu(I) for pMMO. The more widespread occurrence of cytosolic Csps complicates the current conceptually simplistic idea that these organisms have evolved not to use copper in this compartment to help avoid toxicity. A role for these proteins in Cu(I) storage is currently the most logical suggestion for their function, but in many cases what they are storing copper for remains unknown. The presence of bacterial copper storage proteins seems consistent with a number of other observations as follows: 1) that bacterial copper-import systems exist (6, 7, 17, 21, 52, 56, 77, 95), including into the cytosol; 2) that endogenous pools of the metal are available in bacteria (11, 15, 16, 18, 96); and 3) that *E. coli* grown in both LB and minimal medium accumulates copper (97). It also suggests that there are alternative mechanisms to using different cellular compartments to prevent mismetallation of proteins by copper (37). Furthermore, the ability of bacteria to store copper in the cytosol could provide further insight into the observation that certain periplasmic proteins are loaded with copper that has passed through the cytosol (88, 98, 99). Most of the organisms in which this has been reported possess a Csp3.

A lot more work is needed to understand copper storage and removal for the exported Csp1s and the cytosolic Csp3s. Csps are only found in ~40% of methanotrophs, whereas pMMO is nearly always present, and although Csp3s are widespread in bacteria, they are far from ubiquitous. The discovery of the Csps leads to the intriguing question of whether there are other bacterial copper-storage systems yet to be found. Even if this is not the case, the presence of Csp3s indicates that as predicted for other metalloproteomes (100), the possibility exists that there are cytoplasmic copper-requiring enzymes yet to be discovered.

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